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Abstract

We demonstrate that synuclein-gamma (SNCG) also named BCSG1 is abnormally expressed in a high percentage (67.5%) of tumor tissues of diversified cancer types including liver, esophagus, colon, gastric, lung, prostate, cervical, and breast cancer but rarely expressed in tumor matched non neoplastic adjacent tissues (NNAT) (0.6%). Expressions of SNCG protein in different cancer types all display stage-specific patterns of very low expression in stage I and high expression in stages II-IV. Importantly, we observe a strong association between SNCG protein expression in primary tumors with distant metastasis in patients regardless of the cancer type (60.6%, p<0.001). By performing genomic sequencing and methylation-specific PCR assays, we identify an inclusive demethylation of CpG sites within the CpG island of *SNCG* gene in every tumor sample (100%) across all cancer types, illustrating a universal loss of the epigenetic control of *SNCG* gene expression in tumors and further demonstrating that the demethylation of *SNCG* CpG island is primarily responsible for the aberrant expression of SNCG protein in cancerous tissues. These new findings strongly suggest that reactivation of *SNCG* gene expression by DNA demethylation is a common critical contributing factor to malignant progression of many solid tumors and its expression in primary carcinomas is an effective molecular indicator of distant metastasis.

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INTRODUCTION

There is growing evidence that links DNA methylation to the development of human cancer (1-4). DNA hypermethylation to repress transcription of genes encoding tumor suppressors (5,6), cell cycle regulators (7), DNA repair enzymes (8,9), and hormone receptors (10) has been well documented and is being actively studied. However, it is much less known about the loss of this epigenetic control to reactivate transcription of tissue-restricted genes in the development of human cancer.

Synuclein-gamma (SNCG), also named BCSG1 (Breast Cancer Specific Gene 1) is a member of a neuronal protein family synuclein, consisting of synuclein-alpha (SNCA), synuclein-beta (SNCB), and SNCG (11-13). Although this group of proteins is abundantly expressed in brain tissues, their normal cellular functions have not been clearly defined, which is in contrast to the well-known pathological roles of synucleins in a number of human diseases. SNCA is the major component of Lewy bodies in sporadic Parkinson's disease and in a subtype of Alzheimer's disease (14,15). Mutations of SNCA have been detected in several familial cases of Parkinson's disease (16). SNCA peptide also has been identified as the nonamyloid component of amyloid deposition, the hallmark of Alzheimer's disease (17,18). Interestingly, SNCG shares 54% sequence identity with SNCA (19), SNCG is not clearly involved in neurodegenerative diseases. Instead, SNCG has been implicated in human neoplastic diseases, particularly in breast cancer and ovarian cancer. Several studies have shown that SNCG was abnormally expressed in a high percentage of advanced and metastatic breast tumors but not in normal or benign breast tissues (19-21). When overexpressed, SNCG stimulates proliferation and induces metastasis of breast cancer cells (22-26). Analysis of breast tumor samples did not identify any sequence variation of *SNCG* gene from its original normal neuronal environment and no gene amplification was detected either (27), suggesting that transcriptional activation could account for its abundant expression in breast cancer cells. By analyzing the promoter region of *SNCG* gene and conducting genomic sequencing we had demonstrated that the loss of methylation control in a CpG island located in exon 1 of *SNCG* was primarily responsible for the aberrant expression of this neuronal protein in breast carcinoma (28) and in some ovarian carcinomas (29). Further investigations aimed to elucidate the oncogenic functions of this protein have revealed that SNCG overexpression in breast cancer cells resulted in a compromised mitotic checkpoint (30), increased resistance of tumor cells to anti-microtubule drugs (31), and accelerated rate of chromosomal instability (32). Since the mitotic checkpoint control is critical for every cell type to maintain its genetic stability (33,34), the inhibitory effects of SNCG on mitotic checkpoint function imply that the abnormal expression of SNCG in human tissues outside the neuronal system could have general tumorigenic effects and SNCG may play a profound oncogenic role in human cancers beyond breast or ovarian carcinogenesis. However, SNCG protein expression and methylation status of the CpG island in other human cancers have not been carefully examined. Furthermore, the initial observation of augmentation of tumor cell metastasis by SNCG expression obtained in an animal model of breast cancer (22) has not been corroborated by clinical evidence.

In the current study, we independently examined the protein expression and the methylation status of *SNCG* gene in 320 patient samples of malignant and matched non neoplastic adjacent tissues (NNAT) derived from 8 diversified cancer types, including male-specific prostate cancer, female-specific cervical cancer, 4 cancer types in digestive system (liver, esophagus, stomach, colon) and an respiratory specific cancer (lung cancer); patient samples of breast

carcinoma were also included in this study to serve as positive controls. The relationships between SNCG protein expressions with all clinicopathological features of cancer patients were further analyzed in great details to identify significant correlations.

BODY
Specific expressions of SNCG protein in different malignant tumor tissues but not in normal counterparts

Archived tumor tissues and the matched non neoplastic adjacent tissues from 160 patients diagnosed with a variety of cancer types were examined for SNCG protein expression by immunohistochemistry (IHC). Out of 160 tumor samples, 108 (67.5%) samples displayed clear intracellular staining of SNCG protein exclusively in their malignant cells at different expression levels and with a frequency that varied by cancer type: liver (19/20), esophagus (16/20), prostate (15/20), cervical (14/20), gastric (13/20), colon (12/20), breast (10/20), and lung (9/20). By a sharp contrast, only 4 out of 160 tissue samples adjacent to tumors from the same patient cohort showed low immunoreactivity with anti-SNCG antibody (0.6%). Normal tissues of liver, esophagus, prostate, cervix, stomach, and breast of healthy subjects were all negative in IHC of SNCG. These results provide the first evidence for predominant expressions of SNCG protein in a wide range of human cancers but not expressed in their normal counterparts.

Detection of demethylation of SNCG CpG island in malignant and non neoplastic adjacent tissues

To determine whether SNCG expressions in tumor tissues correlate with the demethylation of *SNCG* gene, the methylation status of the CpG island of *SNCG* in tumor and NNAT samples was examined by a nested, two-step MSP assay in combination with direct in vivo sequencing of bisulfite modified genomic DNA isolated from paraffin-embedded fixed tissues of the same patient cohort. Initially, to verify the specificity of the primer pairs that distinguish the CpG sites from unmethylated TpG dinucleotides within the CpG island, the methylated and unmethylated PCR products from one pair of liver tumor/NNAT and one pair of lung tumor/NNAT samples were cloned into pCR2.1 TOPO cloning vector. After transformation, plasmid DNAs isolated from multiple clones of each DNA sample were sequenced. The CpG sites were shown methylated in all clones amplified using methylated primers and were converted to TpG dinucleotides in clones amplified using demethylated primers. With this solid validation of primer specificity and PCR conditions, the MSP method was used to examine the *SNCG* CpG island in 320 DNA samples of tumor and NNAT blindly without knowing the expression status of SNCG protein. Demethylated PCR product of *SNCG* CpG island, either as the sole form or as the predominant form when compared with the methylated PCR product from the same DNA sample, was detected in every tumor sample across all cancer types (100%), indicating a universal loss of the epigenetic control of SNCG gene in tumors. In addition to tumor samples, the 4 NNAT samples that were shown positive in IHC examination with anti-SNCG antibody also contain demethylated *SNCG* gene. These results clearly demonstrate that the demethylation of *SNCG* CpG island is primarily responsible for the aberrant expression of SNCG protein in many solid tumors.

In addition to changes observed in tumor tissues, MSP detected demethylated alleles of *SNCG* in a substantial population of NNAT samples in a tumor-type specific manner. In lung, prostate, and gastric cancers, the *SNCG* CpG island remained predominantly methylated and

demethylation rarely occurred in NNAT samples. The frequency of demethylation in NNAT samples was modest in esophagus and colon cancer. However, significant percentages (40-45%) of NNAT samples of liver, breast, and cervical cancer patients contained demethylated gene at relatively high abundance. By performing exact Chi-square test to evaluate the relationship between SNCG protein expression with the methylation status, we found a close association between the SNCG protein expression in tumor samples with the demethylation of *SNCG* CpG island in tumor adjacent non neoplastic tissues ($p<0.001$). Among 108 tumor samples that expressed SNCG protein, partial demethylation occurred in 45 samples of their non malignant counterparts (41.7%). In contrast, SNCG demethylation was detected only in 3 out of 52 NNAT samples (5.7%) to which their matched tumor samples were negative in SNCG protein expression.

Assessment of methylation patterns of SNCG CpG island in different tissues by genomic sequencing

In order to obtain detailed methylation versus demethylation patterns of *SNCG* CpG island in different malignant and non malignant tissues, with the guidance of MSP results, modified DNAs of two pairs of patient samples from each cancer type that displayed demethylated *SNCG* in tumor and methylated *SNCG* in matched NNAT were selected for genomic sequencing. The results show that nearly all CpG sites within the CpG island of *SNCG* were demethylated in all cancer types; conversely, almost all CpG sites were remained methylated in the matched normal tissues with an exception of normal breast tissues that showed a pattern of partial demethylation at certain CpG sites, consistent with our previous findings (28,29). From these sequencing results we conclude that *SNCG* CpG island is fully methylated in normal tissues of liver, esophagus, prostate, cervix, stomach, colon, and lung but partially methylated in breast tissue. Tumors from these tissues contain completely demethylated *SNCG*.

There are at least two possible reasons to explain the appearance of demethylated alleles in some NNAT samples, particularly in those samples that their malignant counterparts expressed SNCG protein. One possibility is that a small amount of tumor cells might have infiltrated into adjacent tissues and contributed to the demethylated SNCG alleles appeared in NNAT samples. The alternative possibility is that the demethylation event of *SNCG* CpG island precedes malignant transformation in the tumor neighboring cells. To examine these possibilities, we performed genomic sequencing to a number of NNAT samples in which demethylated alleles were detected by MSP. The sequencing data in Fig. 3B revealed that unlike tumor tissues where the CpG islands of *SNCG* were completely demethylated, the CpG island of *SNCG* from all NNAT samples were only partially demethylated at certain CpG sites. Completely demethylated alleles were not found at all. These results ruled out the possibility of tumor cell infiltration and provided direct evidence indicating the occurrence of ongoing genetic changes in these tumor neighboring cells that appeared morphologically normal at the time of surgery.

Associations of SNCG protein expression with disease progression and distant metastasis

In a previous study, the expression of SNCG in MDA-MB435 cells resulted in a massive metastasis of breast cancer cells to lung in nude mice (22), indicating a positive role of this oncogene product in metastasis. To obtain direct clinical evidence, we analyzed clinicopathological features of all patients with relationships to SNCG protein expression status. Significant correlations of SNCG protein expression with advanced stages of tumor were observed in all cancer types. In liver, esophagus, prostate, cervical, breast, and lung

cancers, patients with diseases of stages II-IV all expressed SNCG protein (100%). SNCG positivity was also high at 81.2% and 78.5% in gastric and colon cancers. In contrast, SNCG protein was only detected in 20% or less of patient samples with diseases at stage I or stage 0 from esophagus, prostate, breast, gastric, or colon cancers. A relatively higher percentage (50%) of stage I samples from cervical cancer (6/12) and liver cancer (1/2) expressed SNCG protein.

The correlation of SNCG protein expression with lymph node invasion was found in prostate, breast, and lung cancers. The correlations of SNCG protein expression with lymph node invasion in liver, esophagus, gastric, cervical, and colon cancer were not statistically significant due to the lack of sufficient cases of lower stages of patient samples. However, we found that the SNCG protein expression in primary carcinomas of different cancer types were all strongly associated with distant metastasis ($p<0.001$) with an exclusion of cervical cancer, in which tumors were removed from patients before tumor spread. In liver, esophagus, prostate, colon, breast, and lung cancer, patients with distant metastasis all expressed SNCG protein in their primary carcinomas, and 12/14 metastatic gastric patients were also SNCG-positive. Overall, out of 94 SNCG-positive primary carcinomas, metastasis was found in 57 cancer patients; in contrast, only 2 out of 46 SNCG-negative tumor samples were from the patients with metastatic cancers (60.6% vs. 4%, $p<0.001$). These results reveal a strong association between SNCG protein expression and tumor distant metastasis.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of highly sensitive and specific method of nested methylation specific PCR method (NMSP) to determine SNCG methylation status.

REPORTABLE OUTCOMES

- The manuscript describing this work will be published in Cancer Research on the September issue 2005.

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Loss of Epigenetic Control of *Synuclein-γ* Gene as a Molecular Indicator of Metastasis in a Wide Range of Human Cancers

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Abstract

Metastasis is a major contributing factor to poor prognosis in cancer. Reliable and sensitive biomarkers that indicate the development of metastasis of primary tumors would be of great clinical use. In this study, we show that the neuronal protein synuclein-γ (SNCG) is abnormally expressed in a high percentage (67.5%) of tumor tissues of diversified cancer types, including liver, esophagus, colon, gastric, lung, prostate, cervical, and breast cancer, but rarely expressed in tumor-matched nonneoplastic adjacent tissues (0.6%). Expressions of SNCG protein in different cancer types all display stage-specific patterns of very low expression in stage I and high expression in stages II to IV. Importantly, we observe a strong association between SNCG protein expression in primary tumors with distant metastasis in patients regardless of the cancer type (60.6%, $P < 0.001$). By performing genomic sequencing and methylation-specific PCR assays, we identify an inclusive demethylation of CpG sites within the CpG island of *SNCG* gene in every tumor sample (100%) across all cancer types, illustrating a universal loss of the epigenetic control of *SNCG* gene expression in tumors and further demonstrating that the demethylation of *SNCG* CpG island is primarily responsible for the aberrant expression of SNCG protein in cancerous tissues. These new findings strongly suggest that reactivation of *SNCG* gene expression by DNA demethylation is a common critical contributing factor to malignant progression of many solid tumors and its expression in primary carcinomas is an effective molecular indicator of distant metastasis. Our studies also suggest that the methylation status of *SNCG* gene can be used as a sensitive molecular tool in early detections of tumorigenesis. (Cancer Res 2005; 65(17): 1-9)

Introduction

There is growing evidence that links DNA methylation to the development of human cancer (1–4). DNA hypermethylation to repress transcription of genes encoding tumor suppressors (5, 6), cell cycle regulators (7), DNA repair enzymes (8, 9), and hormone receptors (10) has been well documented and is being actively studied. However, the loss of this epigenetic control to reactivate transcription of tissue-restricted genes in the development of human cancer is much less known.

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In the current study, we independently examined the protein expression and the methylation status of *SNCG* gene in 320

patient samples of malignant and matched nonneoplastic adjacent tissues derived from eight diversified cancer types, including male-specific prostate cancer, female-specific cervical cancer, four cancer types in digestive system (liver, esophagus, stomach, colon), and a respiratory-specific cancer (lung cancer); patient samples of breast carcinoma were also included in this study to serve as positive controls. The relationships between SNCG protein expressions with all clinicopathologic features of cancer patients were further analyzed in great details to identify significant correlations.

Materials and Methods

Tissue specimens. With the Institutional Review Board approval, 160 paraffin-embedded formalin-fixed blocks of tumor tissue and 160 blocks of matched nonneoplastic adjacent tissue were obtained from the Second Hospital of Nanjing City (Nanjing, China) and were H&E counterstained. These samples were derived from 160 patients diagnosed with different types of cancer, including liver, esophagus, prostate, gastric, colon, cervical, lung, and breast cancers, at a frequency of 20 cases per cancer type. Tumors were staged following standard American Joint Committee on Carcinoma (AJCC)/International Union against Carcinoma (UICC) tumor-node-metastasis (TNM) methodology.

Immunohistochemistry. Tissue samples were stained within 1 week of sectioning. Slides of 4 μm sections were deparaffinized with xylene. Following rehydration in distilled water, antigen retrieval was accomplished by heat with EDTA (pH 8.0; Zymed, San Francisco, CA). Endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide at room temperature for 5 minutes. Nonspecific antibody binding was blocked with 5% goat serum for 10 minutes at room temperature. Slides were then incubated with goat anti-SNCG polyclonal antibody (sc-10699, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:300 dilution at 4°C overnight. Following three washes with PBS, slides were incubated with biotin-labeled rabbit anti-goat IgG (Histostain-Plus kit, 50-232, Zymed) for 30 minutes at 37°C. After three washes of PBS, the staining was accomplished by using 3,3'-diaminobenzidine + Substrate Chromogen Systems (DAKO Corp., Carpinteria, CA). Sections were counterstained with hematoxylin, dehydrated, and mounted. Positive cases were defined by the presence of intracellular staining with red/brown color in malignant cells, as seen in positive controls (paraffin sections from stage III breast cancer patients; ref. 20). Negative cases were defined by the absence of specific intracellular

staining, as seen in negative controls, consisting paraffin sections from normal tissues of liver, esophagus, colon, breast, stomach, cervix, and prostate.

A semiquantitative scoring system based on the average number of SNCG-positive cells from five randomly chosen fields of ×400 was used to grade the expression levels regardless of the staining intensity. The mean value (n) was used to grade the expression levels: +, $0 < n \leq 30$; ++, $30 < n \leq 50$; +++, $50 < n \leq 80$. Samples were evaluated under light microscopy independently by two pathologists without prior knowledge of the patients' clinical data.

Nested methylation-specific PCR and genomic sequencing of bisulfite-modified DNA. Fifteen 10 μm consecutive sections were cut from each formalin-fixed, paraffin-embedded tissue block and were incubated with 1 mL xylene at 40°C for 2 hours, washed with ethanol, and centrifuged to remove the supernatant. The procedure of deparaffinization was repeated once and tissues were dried before DNA extraction. DNA was isolated by using DNA isolation kit (Promega) following the protocol of the manufacturer. After elusion from the DNAeasy Mini spin column, samples were dried by lyophilization. For each sample, genomic DNA was resuspended in 30 μL Tris-EDTA buffer and 15 μL DNA was diluted by distilled water to a volume of 50 μL and was denatured by NaOH for 15 minutes at 37°C, followed by the treatment of sodium bisulfite at 50°C for 16 hours (35). The modified DNA was purified using DNA cleanup kit (Promega) in a total volume of 20 μL, and 4 μL were used for genomic sequencing and nested methylation-specific PCR (MSP). Sequences of primers used in this study are listed in Table 1. For the first-step PCR, the modified DNA was amplified with primer SNCG-S2F and SNCG-S2R covering the region −275 to +140 that includes the entire CpG island with 15 CpG sites. PCR reactions were done in a volume of 25 μL containing 1× PCR buffer, 1× TaqMaster PCR Enhancer, 0.125 mmol/L deoxynucleotide triphosphate, 25 pmol of each primer, and 1.25 units of platinum Taq polymerase (Eppendorf). The reactions were carried out at 94°C for 1 minute to activate the hot start enzyme, then 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Subsequently, the first-step PCR product in the reaction tube was diluted 1:10 and 4 μL was used as DNA template for the nested PCR reactions for genomic sequencing and for MSP. For genomic sequencing, the PCR using the primer SNCG-S5F and SNCG-S5R was conducted for 30 cycles with the annealing temperature of 60°C. The 361 bp PCR product covering the region −232 to +129 was gel purified and ligated into pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA). After transformation, plasmid DNAs were isolated from individual colonies and

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Table 1. Primers of *SNCG* for genomic bisulfite sequencing and MSP

Primer	Sequence	Annealing temperature (°C)	Product size (bp)
Bisulfite sequencing PCR primers			
First PCR			
BCSG-S2F	GGTTGAGTTAGTAGGAGTTTA	58	415 (−275 to +140)
BCSG-S2R	CCTACCATACCCCACTTACCC		
Nested PCR			
BCSG1-S5F	TATTTTGGAGGAAGGTGAGGTTG	60	361 (−232 to +129)
BCSG1-S5R	CCACTTACCCACATACATAACC		
MSP primers			
Methylated			
BCSG-M1F	TCGTATTAATATTTTATCGGCGT	60	196 (−141 to +52)
BCSG-M1R	CCGCACCCACCACGCCCTCCTTAACGA		
Unmethylated			
BCSG-U5F	TTGGTGTTAATAGGAGGTATTGGGGA TAGTTGTTGTG	59	180 (−130 to +50)
BCSG-U5R	CACACCCACCACACCCTCCTTAACAAT		
WT primers			
SNCG-WF	ACGCAGGGCTGGCTGGGCTCCA	60	242 (−169 to +72)
SNCG-WR	CCTGCTTGGTCTTTTCCACC		

Figure 1. Representative immunohistochemical staining for SNCG protein in human carcinomas and in matched nonneoplastic adjacent tissues. For each sample, numbers of SNCG-positive cells were counted from five randomly chosen fields of ×400 and averaged. The mean value (n) was used to grade the expression levels: +, $0 < n \leq 30$; +, $30 < n \leq 50$; +++, $50 < n \leq 80$. A, a positive staining at a level of +++ of a cervical carcinoma. B, a negative staining of the matched adjacent normal cervical tissue. C, a positive staining at a level of +++ of a gastric carcinoma. D, a negative staining of the matched adjacent normal gastric tissue. E, a positive staining at a level of ++ of a colon carcinoma. F, a negative staining of the matched adjacent normal colon tissue. G, a positive staining at a level of + of a prostate carcinoma. H, a negative staining of the matched adjacent normal prostate tissue.
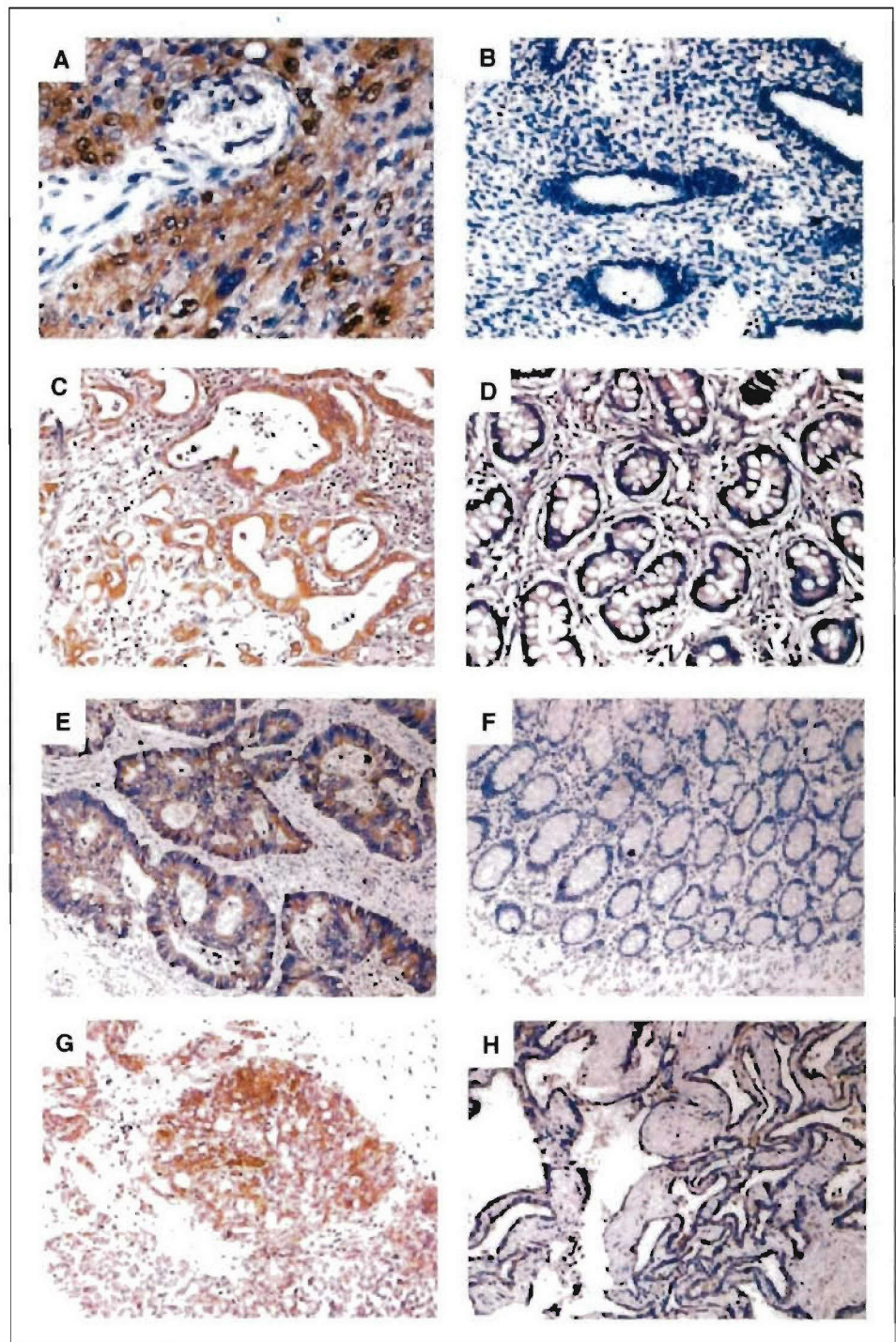
subjected to sequencing using M13 as sequencing primer to obtain the entire map of *SNCG* CpG island.

For nested MSP, the primer SNCG-M1F and SNCG-M1R were used to amplify methylated *SNCG* alleles for 25 cycles at the annealing temperature of 60°C to yield a PCR product of 196 bp that includes 13 of 15 CpG sites of the CpG island. Unmethylated alleles were amplified with primer SNCG-U5F and SNCG-U5R at the annealing temperature of 59°C for 25 cycles, which yields a PCR product of 180 bp that covers 12 of 15 CpG sites of the CpG island. The final PCR products of the nested MSP were separated on 2% agarose gels, stained with ethidium bromide, and photographed by Kodak Imaging Station

400. The intensity of each band was quantified and the relative abundance of unmethylated band over total amount of PCR products, including methylated and unmethylated for each DNA sample, was expressed as percentage of total. One pair of PCR products of lung tumor/nonneoplastic adjacent tissue and one pair of PCR products of liver tumor/nonneoplastic adjacent tissue were also gel purified and ligated into pCR2.1-TOPO cloning vector for sequencing to validate the specificity of the MSP method.

Statistical analysis. The probabilities of SNCG protein expression, distant metastasis, as well as nonneoplastic adjacent tissue demethylation for the overall patients were compared between different sample groups (tumor

tissue versus nonneoplastic adjacent tissue; SNCG protein positive versus SNCG protein negative) by means of the χ^2 test. The probability of SNCG protein expression, stages, as well as lymph node invasion for each cancer type were compared between tumor tissue and nonneoplastic adjacent tissue using Fisher's exact test with correction for continuity, as the sample size was too small to use the normal approximation to the binomial distribution.

Results

Specific expressions of SNCG protein in different malignant tumor tissues but not in normal counterparts. Archived tumor tissues and the matched nonneoplastic adjacent tissues from 160 patients diagnosed with a variety of cancer types were examined for SNCG protein expression by immunohistochemistry. Out of 160 tumor samples, 108 (67.5%) samples displayed clear intracellular staining of SNCG protein exclusively in their malignant cells at different expression levels and with a frequency that varied by cancer type: liver (19 of 20), esophagus (16 of 20), prostate (15 of 20), cervical (14 of 20), gastric (13 of 20), colon (12 of 20), breast (10 of 20), and lung (9 of 20; Fig. 1; Table 2). By a sharp contrast, only 4 of 160 tissue samples adjacent to tumors from the same patient cohort showed low immunoreactivity with anti-SNCG antibody (0.6%). Normal tissues of liver, esophagus, prostate, cervix, stomach, and breast of healthy subjects were all negative in immunohistochemistry of SNCG. These results provide the first evidence of predominant expressions of SNCG protein in a wide range of human cancers but not expressed in their normal counterparts.

Detection of demethylation of SNCG CpG island in malignant and nonneoplastic adjacent tissues. To determine whether SNCG expressions in tumor tissues correlate with the demethylation of SNCG gene, the methylation status of the CpG island of SNCG in tumor and nonneoplastic adjacent tissue samples was examined by a nested, two-step MSP assay in combination with direct *in vivo* sequencing of bisulfite-modified genomic DNA isolated from paraffin-embedded fixed tissues of the same patient cohort. Figure 2A (*top*) shows a diagram of the CpG island and corresponding regions of MSP products using methylated as well as unmethylated SNCG primers. Initially, to verify the specificity of the primer pairs that distinguish the CpG sites from unmethylated TpG dinucleotides within the CpG island, the methylated and unmethylated PCR products from one pair of liver tumor/nonneoplastic adjacent tissue and one pair of lung tumor/nonneoplastic adjacent tissue samples were cloned into pCR2.1 TOPO cloning vector. After transformation, plasmid DNAs isolated from multiple clones of each DNA sample were sequenced. The CpG sites were shown methylated in all clones amplified using methylated primers and were converted to TpG dinucleotides in clones amplified using demethylated primers (Fig. 2A, *bottom*). With this solid validation of primer specificity and PCR conditions, the MSP method was used to examine the SNCG CpG island in 320 DNA samples of tumor and nonneoplastic adjacent tissue blindly, without knowing the expression status of SNCG protein. Representative results of five pairs of DNA samples from each cancer type are shown in Fig. 2B-D and all results are summarized in Table 2. Demethylated PCR product of SNCG CpG island, either as the sole form or as the predominant form when compared with the methylated PCR product from the same DNA sample, was detected in every tumor sample across all cancer types (100%), indicating a universal loss of the epigenetic control of SNCG gene in tumors. In addition to tumor samples, the four nonneoplastic adjacent tissue samples that were shown positive in immunohistochemistry examination with anti-SNCG antibody also contain demethylated

Table 2. SNCG protein is highly expressed in tumor tissues but not in nonneoplastic adjacent tissues and the expression is correlated with demethylation of the CpG island of SNCG gene

Tissues	Sample no.	Protein expression*	Gene demethylation[†]
Liver			
Tumor	20	19 (+,3; ++,5; +++,11)[‡]	20[‡]
NNAT	20	0	9
Esophagus			
Tumor	20	16 (+,4; ++,4; +++,6)[‡]	20[‡]
NNAT	20	0	7
Gastric			
Tumor	20	13 (+,1; ++,4; +++,8)[‡]	20[‡]
NNAT	20	0	3
Cervix			
Tumor	20	14 (+,7; ++,3; +++,1)[‡]	20[‡]
NNAT	20	0	8
Breast			
Tumor	20	10 (+,6; ++,2; +++,2)[‡]	20[‡]
NNAT	20	1 (+,1)	9
Colon			
Tumor	20	12 (++,6; ++,4; +++,2)[‡]	20[‡]
NNAT	20	1 (+,1)	7
Prostate			
Tumor	20	15 (+,10; ++,5)[‡]	20[‡]
NNAT	20	2 (+, 2)	4
Lung			
Tumor	20	9 (+,1; ++,4; +++,1)[‡]	20[‡]
NNAT	20	0	2
Σ			
Tumor	160	108[‡]	160[‡]
NNAT	160	4	49

Abbreviation: NNAT, nonneoplastic adjacent tissue.
*Protein expression assessed by immunohistochemistry. For each sample, numbers of SNCG-positive cells were counted from five randomly chosen fields of ×400 and averaged. The mean value (n) was used to grade the expression levels: +, $0 < n \leq 30$; ++, $30 < n \leq 50$; +++, $50 < n \leq 80$.
[†]SNCG gene demethylation assessed by MSP.
[‡]$P < 0.001$.

SNCG gene. These results clearly show that the demethylation of SNCG CpG island is primarily responsible for the aberrant expression of SNCG protein in many solid tumors.
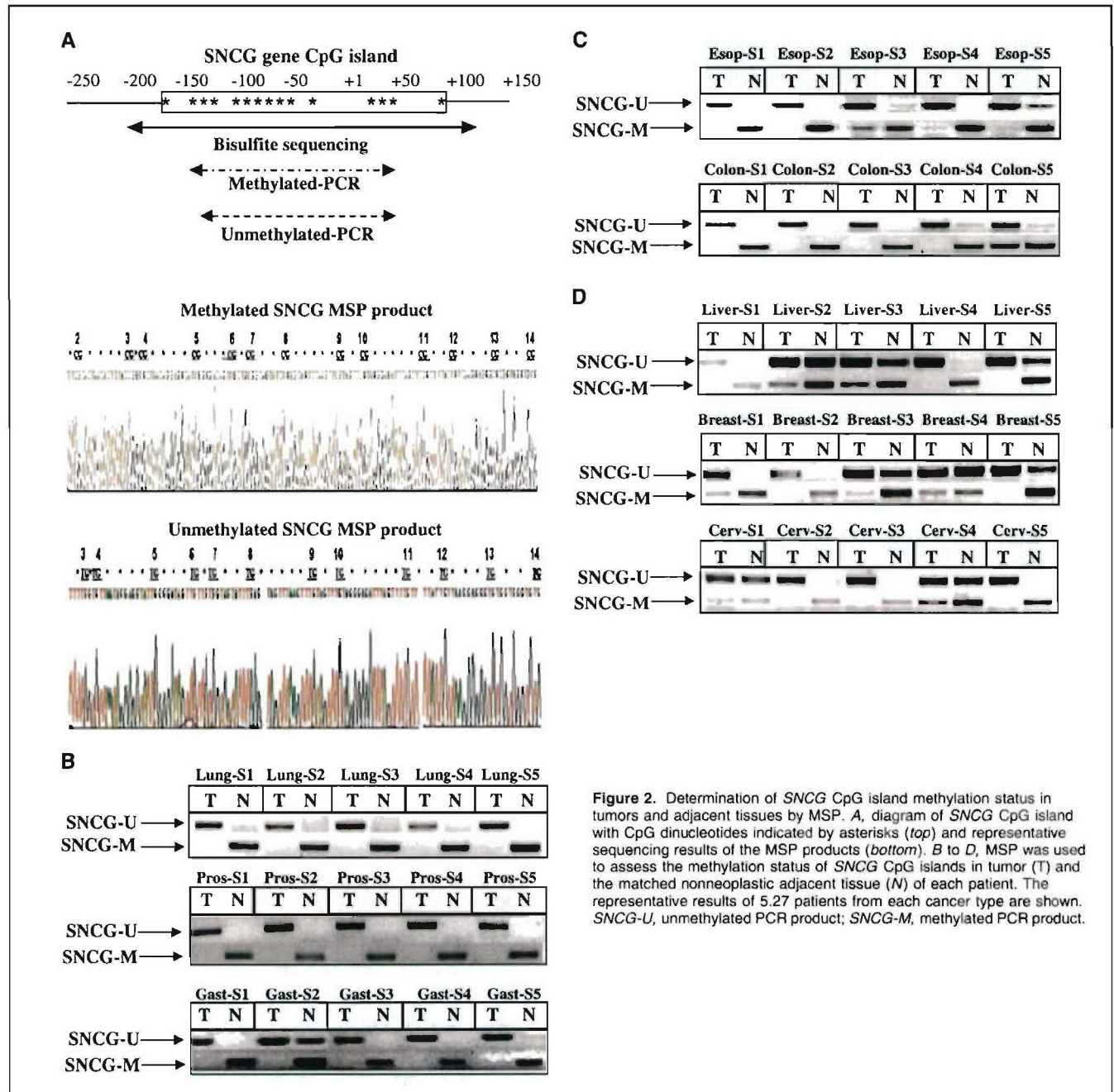
In addition to changes observed in tumor tissues, MSP-detected demethylated alleles of SNCG in a substantial population of nonneoplastic adjacent tissue samples in a tumor type–specific manner. In lung, prostate, and gastric cancers, the SNCG CpG island remained predominantly methylated and demethylation rarely occurred in nonneoplastic adjacent tissue samples (Fig. 2B). The frequency of demethylation in nonneoplastic adjacent tissue samples was modest in esophagus and colon cancer (Fig. 2C). However, significant percentages (40-45%) of nonneoplastic adjacent tissue samples of liver, breast, and cervical cancer patients contained demethylated gene at relatively high abundance (Fig. 2D). By performing exact χ^2 test to evaluate the relationship between SNCG protein expression with the methylation status, we found a close association between the SNCG protein expression

in tumor samples with the demethylation of *SNCG* CpG island in tumor adjacent nonneoplastic tissues ($P < 0.001$). Among 108 tumor samples that expressed SNCG protein, partial demethylation occurred in 45 samples of their nonmalignant counterparts (41.7%). In contrast, SNCG demethylation was detected only in 3 of 52 nonneoplastic adjacent tissue samples (5.7%) to which their matched tumor samples were negative in SNCG protein expression.

Assessment of methylation patterns of SNCG CpG island in different tissues by genomic sequencing. To obtain detailed methylation versus demethylation patterns of *SNCG* CpG island in different malignant and nonmalignant tissues, with the guidance of MSP results, modified DNAs of two pairs of patient samples from

each cancer type that displayed demethylated *SNCG* in tumor and methylated *SNCG* in matched nonneoplastic adjacent tissue were selected for genomic sequencing (Fig. 3*A*). Nearly all CpG sites within the CpG island of *SNCG* were demethylated in all cancer types; conversely, almost all CpG sites were remained methylated in the matched normal tissues with an exception of normal breast tissues that showed a pattern of partial demethylation at certain CpG sites, consistent with our previous findings (28, 29). From these sequencing results, we conclude that *SNCG* CpG island is fully methylated in normal tissues of liver, esophagus, prostate, cervix, stomach, colon, and lung but partially methylated in breast tissue. Tumors from these tissues contain completely demethylated *SNCG*.

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Figure 2. Determination of *SNCG* CpG island methylation status in tumors and adjacent tissues by MSP. *A,* diagram of *SNCG* CpG island with CpG dinucleotides indicated by asterisks (*top*) and representative sequencing results of the MSP products (*bottom*). *B* to *D,* MSP was used to assess the methylation status of *SNCG* CpG islands in tumor (T) and the matched nonneoplastic adjacent tissue (N) of each patient. The representative results of 5.27 patients from each cancer type are shown. *SNCG-U,* unmethylated PCR product; *SNCG-M,* methylated PCR product.
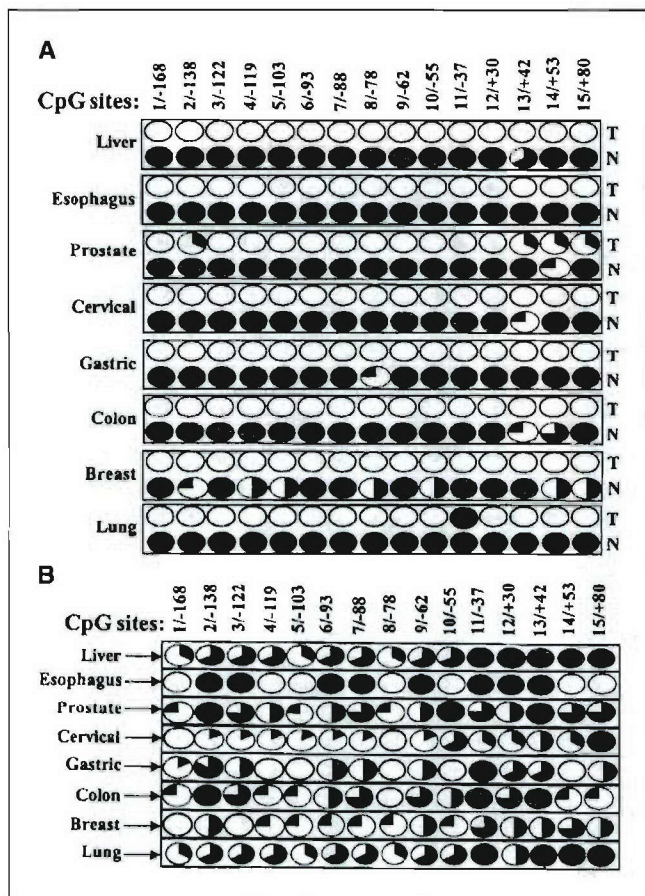
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Figure 3. Methylation patterns of CpG island of *SNCG* in various tumor and matched nonneoplastic adjacent tissues. CpG positions are indicated relative to the translation start codon; each circle in the figure represents a single CpG site. For each DNA sample, the percentage of demethylation at a single CpG site is calculated from the sequencing results of six to eight independent clones. ○, 100% demethylation; ●, 0% demethylation. In (*A*), DNAs of two pairs of patient samples from each cancer type that displayed demethylated *SNCG* in tumor (*T*) and methylated *SNCG* in matched nonneoplastic adjacent tissue (*N*) was selected for genomic sequencing; the selected tumor samples were negative in SNCG protein expression. In (*B*), DNAs of two nonneoplastic adjacent tissue samples that displayed demethylated *SNCG* in assays of MSP and their tumor counterparts expressed SNCG protein were selected for each cancer type.

the occurrence of ongoing genetic changes in these tumor neighboring cells that appeared morphologically normal at the time of surgery.

Associations of SNCG protein expression with disease progression and distant metastasis. In a previous study, the expression of SNCG in MDA-MB435 cells resulted in a massive metastasis of breast cancer cells to lung in nude mice (22), indicating a positive role of this oncogene product in metastasis. To obtain direct clinical evidence, we analyzed clinicopathologic features of all patients with relationships to SNCG protein expression status (Table 3). Significant correlations of SNCG **T3** protein expression with advanced stages of tumor were observed in all cancer types. In liver, esophagus, prostate, cervical, breast, and lung cancers, patients with diseases of stages II to IV all expressed SNCG protein (100%). SNCG positivity was also high at 81.2% and 78.5% in gastric and colon cancers. In contrast, SNCG protein was only detected in ≤20% of patient samples with diseases at stage I or stage 0 from esophagus, prostate, breast, gastric, or colon cancers. A relatively higher percentage (50%) of stage I samples from cervical cancer (6 of 12) and liver cancer (1 of 2) expressed SNCG protein.

The correlation of SNCG protein expression with lymph node invasion was found in prostate, breast, and lung cancers. The correlations of SNCG protein expression with lymph node invasion in liver, esophagus, gastric, cervical, and colon cancer were not statistically significant due to the lack of sufficient cases of lower stages of patient samples. However, we found that the SNCG protein expression in primary carcinomas of different cancer types were all strongly associated with distant metastasis (*P* < 0.001) with an exclusion of cervical cancer in which tumors were removed from patients before tumor spread. In liver, esophagus, prostate, colon, breast, and lung cancer, patients with distant metastasis all expressed SNCG protein in their primary carcinomas, and 12 of 14 metastatic gastric patients were also SNCG positive. Overall, of 94 SNCG-positive primary carcinomas, metastasis was found in 57 cancer patients; in contrast, only 2 of 46 SNCG-negative tumor samples were from the patients with metastatic cancers (60.6% versus 4%, *P* < 0.001; Table 4). These results reveal a strong **T4** association between SNCG protein expression and tumor distant metastasis.

Discussion

SNCG gene was previously considered breast cancer specific and was named as breast cancer specific gene 1 (*BCSG1*) due to its high expression in breast carcinomas and its shown ability in inducing breast cancer cells to proliferate and metastasis. In this study, we provide direct evidence that shows a broad expression spectrum of this oncogene product in different cancers, thereby reinforcing the importance of SNCG in human malignancy. Several major new findings of this current study significantly extend the previous understanding of the pathologic role of this neuronal protein in human neoplastic diseases.

We show that SNCG protein is highly expressed in diversified cancer types, including the female hormone–sensitive cervical and breast cancers, male hormone–sensitive prostate cancer, four cancer types of the digestive system, and lung cancer, the leading cause of mortality in both men and women. It is noteworthy that during the course of this investigation, SNCG protein expression in gastric cancer (36) and pancreatic cancer (37, 38) were also detected by independent research groups. Combined with previous

There are at least two possible reasons to explain the appearance of demethylated alleles in some nonneoplastic adjacent tissue samples, particularly in those samples that their malignant counterparts expressed SNCG protein. One possibility is that a small amount of tumor cells might have infiltrated into adjacent tissues and contributed to the demethylated SNCG alleles that appeared in nonneoplastic adjacent tissue samples. The alternative possibility is that the demethylation event of *SNCG* CpG island precedes malignant transformation in the tumor neighboring cells. To examine these possibilities, we did genomic sequencing to a number of nonneoplastic adjacent tissue samples in which demethylated alleles were detected by MSP. The sequencing data in Fig. 3*B* revealed that unlike tumor tissues where the CpG islands of *SNCG* were completely demethylated, the CpG island of *SNCG* from all nonneoplastic adjacent tissue samples were only partially demethylated at certain CpG sites. Completely demethylated alleles were not found at all. These results ruled out the possibility of tumor cell infiltration and provided direct evidence indicating

Table 3. Correlation between SNCG protein expression status and clinicopathologic factors of different cancers

Cancer patients, clinicopathologic factors	No. patients	SNCG protein expression Negative	Positive
Liver cancer			
Sex			
Male	17	1	16
Female	3	0	3
Age (y)		68	46.2 ± 8.3
Stage			
I	2	1	1
II-IV	18	0	18
Lymph node invasion			
Positive	18	0	18
Negative	2	1	1
Distant metastasis			
Positive	13	0	13
Negative	7	1	6
Esophagus cancer			
Sex			
Male	14	3	11
Female	6	1	5
Age (y)		47.5 ± 6.2	55.1 ± 8.1
Stage			
I	5	4	1
II-IV	15	0	15
Lymph node invasion			
Positive	6	0	6
Negative	14	4	10
Distant metastasis			
Positive	6	0	6
Negative	14	4	10
Prostate cancer			
Sex			
Male	20	5	15
Age (y)		73.2 ± 3.1	73.4 ± 6.0
Stage			
I	6	5	1
II-IV	14	0	14
Lymph node invasion			
Positive	14	0	14
Negative	6	5	1
Distant metastasis			
Positive	5	0	5
Negative	15	5	10
Cervical cancer			
Sex	20	6	14
Female			
Age (y)		45.6 ± 8.7	48.7 ± 12.3
Stage			
0-I	12	6	6
II-IV	8	0	8
Lymph node invasion			
Positive	9	1	8
Negative	11	1	10
Distant metastasis			
Positive	0	0	0
Negative	20	6	14
Gastric cancer			
Sex			
Male	17	5	12
Female	3	2	11
Age (y)		52.7 ± 15.0	55.3 ± 10.7
Stage			
I	4	4	0
II-IV	16	3	13
Lymph node invasion			
Positive	20	7	13
Negative	0	0	0
Distant metastasis			
Positive	14	2	12
Negative	6	5	1
Colon cancer			
Sex			
Male	8	3	5
Female	12	5	7
Age (y)		68.2 ± 7.9	63.4 ± 14.1
Stage			
I	6	5	1
II-IV	14	3	11
Lymph node invasion			
Positive	4	0	4
Negative	16	8	8
Distant metastasis			
Positive	4	0	4
Negative	16	8	8
Breast cancer			
Sex			
Female	20	10	10
Age (y)		49.7 ± 10.1	51.5 ± 18.0
Stage			
I	11	10	1
II-IV	9	0	9
Lymph node invasion			
Positive	12	2	10
Negative	8	8	0
Distant metastasis			
Positive	10	0	10
Negative	10	10	0
Lung cancer			
Sex			
Male	16	8	8
Female	4	3	1
Age (y)		59.4 ± 10.7	63.6 ± 11.4
Stage			
I	6	6	0
II-IV	14	5	9
Lymph node invasion			
Positive	14	5	9
Negative	6	6	0
Distant metastasis			
Positive	7	0	7
Negative	13	13	0

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studies in ovarian cancer thus far (20), the abnormal expression of SNCG is clearly linked to 10 different malignant diseases. Although the percentages of SNCG-positive cases differ among different tumor types, the strong association of SNCG expression with higher

Table 4. Association of SNCG protein expression with distant metastasis of tumor cells

Cancer type	No. tumor sample	SNCG protein expression		Distant metastasis
Liver cancer	2	+	19	13
		−	1	0
Esophagus cancer	2	+	16	6
		−	4	0
Gastric cancer	2	+	13	12
		−	7	2
Breast cancer	2	+	10	10
		−	10	0
Colon cancer	2	+	12	4
		−	8	0
Prostate cancer	2	+	15	5
		−	5	0
Lung cancer	2	+	9	7
		−	11	0
Σ	140	+	94*	57 (60.6%)*
		−	46	2 (4%)

NOTE: SNCG protein expressions were assessed by immunohistochemistry and number of positive samples include all expression levels. +, positive of SNCG protein expression by immunohistochemistry; −, negative of SNCG protein expression by immunohistochemistry. Tumors were staged following standard AJCC/UICC TNM methodology.
*$P < 0.001$.

stages of disease is a common feature in this study and in previous reports. Our results are consistent with the notion that SNCG promotes disease progression (19, 22). How SNCG induces disease progression in different cancer types remains elusive. In breast cancer cells, SNCG has been shown to act as a chaperon for estrogen receptor and stimulate estrogen receptor-α signaling pathway that leads to cell proliferation (25, 26). On the other hand, we have shown that the inhibitory effects of SNCG on mitotic checkpoint function are mediated through the mitotic checkpoint kinase BubR1 and are independent of the expression status of estrogen receptor-α (30). Because synucleins have chaperone-like activities (39), it may interact with different proteins in different cellular background. Identifications of specific cellular targets of SNCG in different tumor types will provide insight to delineate its oncogenic functions in human malignancy.

By utilizing the highly specific method of MSP, we show that the CpG island of SNCG is nearly completely demethylated in every tumor tissue examined in this study, illustrating a universal loss of the epigenetic control of SNCG in malignant tumors regardless of the cancer type. To our knowledge, this is the first example of a complete change in methylation status of a cellular gene in multiple cancer types. In most reported studies, specific alterations in methylation status of a CpG island usually occur in certain

percentages of tumors and in limited tumor types (40–45). Thus, detection of fully demethylated alleles of SNCG by the powerful MSP method can be used as a sensitive method in early detection to find a small number of malignant cells in morphologically normal tissues before tumors emerge. The lack of SNCG protein expression in some tumor tissues with demethylated gene suggests that demethylation is necessary but may not be the only factor for reactivating the transcription of this tissue-restricted gene, keeping in line with our previous findings in breast cancer cells (28, 29). In addition, our detection of partially demethylated SNCG alleles in tumor neighboring cells in some patient samples may have important clinical implications. It indicates that the abnormal genetic changes have already initiated in the tumor adjacent tissues that were morphologically normal at the time of surgery. Thus, assessment of the SNCG methylation status can also be applied to determine the genetic abnormality in precancer conditions.

Metastasis is recognized as the most important feature of malignant tumors. Metastatic spread strongly reduces the possibility of cure and survival time. Up to date, no definitive judgment can be made about the probability of metastasis from pathologic examination of the surgically removed primary tumor tissues. Molecular marker–based pathologic indications of metastasis have great potentials in the clinical application (46). Whereas a previous study in nude mice had shown that SNCG expression stimulated breast cancer cells to metastasis (22), the direct clinical evidence of a role of SNCG in tumor metastasis was lacking. Our current studies reveal a strong correlation between SNCG expression in primary tumors and distant metastasis in patients of all cancer types. Our findings not only corroborate the data from animal studies to some extent but also further suggest that SNCG expression status can be considered as a pathologic indication to predict the propensity of metastasis to distant organs and aid in guidance for designing optimized and individualized therapeutic regimens for patients when the tumor samples are available after surgeries.

Taken together, our new findings strongly suggest that the loss of epigenetic control of the neuron-restricted expression of *SNCG* gene is likely a common critical contributing factor to malignant progression of different types of human cancer, and that the strong association of SNCG protein expression in primary carcinomas with distant metastasis marks this protein an effective molecular indicator of tumor metastasis.

Acknowledgments

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